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Quantitation of Minimal Residual Disease in t(8;21)-Positive Acute Myelogenous Leukemia Patients Using Real-Time Quantitative RT-PCR

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t(8;21) is one of the common chromosomal translocations in acute myelogenous leukemia (AML). Using a recently developed real-time quantitative polymerase chain reaction (PCR) system, we analyzed the minimal residual disease (MRD) in bone marrow samples from seven AML patients with t(8;21) at different time points during the clinical courses of their disease. Four of these patients received chemotherapy and allogenic bone marrow transplantation (allo-BMT), and the other three were treated with chemotherapy alone. Two of the patients that received allo-BMT suffered a relapse. In these patients, the levels of AML1-MTG8 mRNA expression were shown to quantitatively increase. After re-induction chemotherapy and donor lymphocyte infusion therapy, AML went into remission and the expression levels decreased. In the other two patients receiving allo-BMT, the disease went into remission and the level of AML1-MTG8 mRNA expression remained under the detectable range. The other three patients received several courses of chemotherapy, without allo-BMT, and all of them clinically reached the hematological and cytogenetic remission state. However, there were low but detectable levels of MRD in their bone marrow samples. These results suggest that the real-time quantitative PCR assay is very useful for the monitoring of MRD and detecting an early relapse. This assay may also be useful in determining the quantitative difference in myelo-ablative activity between the chemotherapy alone and chemotherapy in conjunction with allo-BMT. Am. J. Hematol. 64:101–106, 2000. © 2000 Wiley-Liss, Inc.

Key words: acute myelogenous leukemia; t(8;21); real-time quantitative PCR; minimal residual disease

INTRODUCTION

t(8;21) is one of the most common chromosomal translocations in acute myelogenous leukemia (AML), occurring in about 20% of adult AML subtype M2 [1]. Patients with t(8;21) usually respond well to chemotherapy, with a high remission rate and relatively long median survival [2–5]. In t(8;21), the AML1 gene on chromosome 21 fuses with the MTG8(ETO) gene on chromosome 8, producing the AML1-MTG8 chimera gene which leads to the expression of AML1-MTG8 chimera mRNA [6–14].

The use of the reverse transcription polymerase chain reaction (RT-PCR) assay to detect AML1-MTG8 chimera mRNA has been widely used for the molecular diagnosis of this type of leukemia. However, the detection of minimal residual disease (MRD) by a qualitative PCR method has been considered to have less clinical value for the assessment of a patient's prognosis. Using the qualitative RT-PCR, several studies have detected MRD in AML (M2) patients who remained in hematological and cytogenetic long-term remission after chemotherapy alone or after bone marrow transplantation (BMT) [15–19]. RT-PCR would be more useful if it

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TABLE I. Clinical Status of the Patients With Acute Myelogenous Leukemia (M2)*

Patient no.	Age/sex	Initial treatment (course)[a]	States at BMT	Treatment after relapse	Current status (months from diagnosis)
1	31/M	Chemotherapy (8) + Allo-BMT	Rel 1	DLI (3) + Chemotherapy (2)	Dead, Rel 3 (24)
2	21/F	Chemotherapy (5) + Allo-BMT	Rel 1	DLI (3) + Chemotherapy (1)	Alive, CR 3 (23)
3	44/M	Chemotherapy (3) + Allo-BMT	CR 1	–	Alive, CR 1 (15)
4	21/M	Chemotherapy (7) + Allo-BMT	CR 1	–	Alive, CR 1 (29)
5	23/M	Chemotherapy (10)	–	–	Alive, CR 1 (17)
6	38/M	Chemotherapy (8)	–	–	Alive, CR 1 (14)
7	46/M	Chemotherapy (8)	–	–	Alive, CR 1 (9)

*Allo-BMT, allogeneic bone marrow transplantation; DLI, donor lymphocytes infusion; CR, complete remission; Rel, relapsing state.
[a]All patients received intensive remission induction chemotherapy with daunorubicin, cytarabine, 6-mercaptopurine, and prednisolone, as according to the AML-89 protocol of Japan Adult Leukemia Study Group (Kobayashi et al., 1996) [28].

could quantitatively evaluate the transcripts during the clinical stages to define a threshold level for clinically significant MRD. Recently, competitive PCR assays have been used to quantify the expression of AML1-MTG8 transcripts in AML (M2) [20,21]. However, this method is time-consuming and vulnerable to contamination, and the final results are not always consistent.

In this study, we used a recently developed sensitive and highly reproducible real-time PCR system [22] for the quantitative measurement of AML1-MTG8 mRNA levels. Here with this method, we monitored seven AML (M2) patients treated with chemotherapy alone or in conjunction with allogenic bone marrow transplantation (allo-BMT).

MATERIALS AND METHODS

Patients and Samples

The MRD was monitored in seven patients with acute myelogenous leukemia with t(8;21) (Table I) at different time points during the clinical course. All the patients underwent intensive induction chemotherapy. In addition, four of the patients received allo-BMT. Two of the patients in the allo-BMT group received a donor lymphocyte infusion (DLI) because of a hematological relapse. Diagnosis of AML-M2 was made according to the French-American-British (FAB) morphological and cytochemical criteria. The presence of t(8;21)(q22;q22) was confirmed by karyotype analysis. The existence of the AML1-MTG8 mRNA transcripts at diagnosis was confirmed in all seven cases by a nested RT-PCR method performed by SRL, Inc. (Tokyo, Japan).

Bone marrow or peripheral blood samples were collected at different times during the clinical course. Mononuclear cells from the collected samples were isolated by Ficoll-Paque density gradient centrifugation and either used immediately for RNA isolation or stored at −80°C. Two AML cell lines, t(8;21)-positive SKNO-1 [23] and t(8;21)-negative SKK were used in this study and were cultured in RPMI-1640, supplemented with 10% fetal bovine serum and 10 μg/mL kanamycin sulfate. Total RNA was extracted from the isolated mononuclear cells and cell lines using TRIzol (Life Technologies, Inc., Gaithersburg, MD). The resulting RNA was used for cDNA preparation.

PCR Conditions, Threshold Cycles, and Controls

Each 1 μg of RNA was reverse transcribed using random hexamers and a SuperScript pre-amplification system (Life Technologies) following the manufacturer's protocol. An aliquot of 1/4th of the resulting cDNA was used for quantitative PCR amplification.

To evaluate the expression of AML1-MTG8 in the samples, quantitative PCR was performed using a Sequence Detector (ABI PRISM 7700, Perkin-Elmer Applied Biosystems, Foster City, CA). Selection of primers and probes for AML1-MTG8 was performed by using Primer Express software (Perkin-Elmer Applied Biosystems). AML1-MTG8 forward primer is located in exon 5 in AML1 gene [24], and the reverse primer is located in exon 2 in MTG8 gene [25] (Fig. 1A). The AML1-MTG8 probe spans the fusion point [11]. The sequences of the forward (F) and reverse (R) primers and probe for GAPDH were as follows according to the manufacturer's manual: GAPDH F: 5′-GAAGGTGAAGGTCGGAGTC-3′, GAPDH R: 5′-GAAGATGGTGATGGGATTTC-3′, GAPDH probe: 5′-CAAGCTTCCCGTTCTCAGCC-3′. The used TaqMan probes consisted of an oligonucleotide with a 5′ FAM (6-carboxyfluorescein) reporter dye and a 3′ quencher dye, TAMRA (6-carboxytetramethylrhodamine).

Real-time PCR was done according to the manufacturer's manual. It was based upon the TaqMan assay and used a fluorogenic oligonucleotide probe labeled with both a labeled fluorescent dye and a quencher dye. In the intact TaqMan probe, the 5′ fluorescent reporter dye was quenched by the 3′ quencher dye through a Foster-type energy transfer. Fluorogenic DNA probes (TaqMan probes), after hybridizing to the template DNA, were hydrolyzed by 5′ secondary structure-dependent nuclease activity of the Taq DNA polymerase. After hydrolysis, the release of the reporter signal caused an increase in

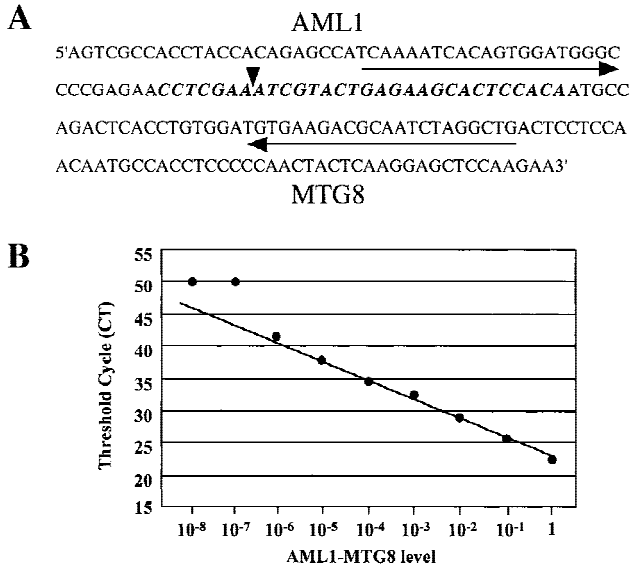

Fig. 1. Real-time PCR assay system for AML1-MTG8 transcripts. (A) localization of PCR primers and probe in AML-MTG8 fusion transcript sequences. Primer sequences are underlined by arrows, and probe sequences are italicized. An arrowhead indicates the AML1-MTG8 fusion point. (B) Standard curve for AML1-MTG8 transcripts. A linear pattern was found between the amount of AML1-MTG8 transcripts and the threshold cycle (CT). This curve was used to calculate the level of AML1-MTG8 transcripts for unknown sample.

fluorescence intensity that was proportional to the accumulation of the PCR product. The fluorescence intensity of the reporter label was normalized using the rhodamine derivative ROX as a passive reference label present in the buffer solution. The system generates a real-time amplification plot based upon the normalized fluorescence signal. Subsequently the threshold cycle (CT) was determined, for example, the cycle number at which the amount of amplified target reached a fixed threshold. The fixed threshold was usually set at 10 standard deviations above the mean of baseline emission calculated from cycles 1 to 15. The CT was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparing of the CTs of a serial dilution of a positive control.

Each 1/20th of the corresponding cDNA was used for quantitative PCR in a 50 μL volume using Master Mix, which includes PCR buffer, $MgCl_2$, dATP, dCTP, dGTP, dUTP, AmpErase UNG, and AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems). Thermal cycling conditions were as recommended by the manufacturer (15 s at 95°C and 1 min at 60°C), with an initial 2 min at 50°C and a final 10 min at 95°C. Fifty cycles were used for AML1-MTG8 expression, and 40 cycles were used for GAPDH expression.

Real-Time Quantitation

The t(8;21)-positive leukemic cell line SKNO-1 was used for a positive control and t(8;21)-negative leukemic cell line SKK was used for a negative control. For the construction of a standard curve of positive controls, serial 10-fold dilutions of total RNA from t(8;21)-positive leukemic cell line SKNO-1 were used for the expression of AML1-MTG8. Quantitation of the expressed AML1-MTG8 of each sample was evaluated according to the simultaneously plotted standard curve. Each sample was normalized to the expression of GAPDH which was performed using the corresponding cDNA of the same samples.

Definition of Remission

A patient was considered to be in hematological remission when less than 5% of the cells in a cellular marrow specimen were blasts. Cytogenetic remission was defined as a hematological remission associated in conjunction with the disappearance of the t(8;21)(q22;q22). If the AML1-MTG8 expression level was under the detectable range using real-time PCR, the patient was considered to be in a molecular remission state.

RESULTS

Sensitivity of the Real-Time Quantitative PCR

To assess the sensitivity and accuracy of the real-time quantitative PCR system, RNA of the AML1-MTG8-positive cell line SKNO-1 was serially diluted with RNA of the AML1-MTG8-negative cell line SKK and cDNA was prepared from the serial dilutions. The resulting cDNA was used for the PCR amplification of the AML1-MTG8 expression. The level of expression obtained from the undiluted SKNO-1 RNA was defined arbitrarily as 1. We detected AML1-MTG8 mRNA up to a dilution level of 10^{-6} and a standard curve was made based on the expression levels for the dilutions (Fig. 1B).

AML1-MTG8 mRNA Levels in Patients Who Received Chemotherapy With Allo-BMT

Figure 2 shows the quantitative levels of the AML1-MTG8 expression normalized to the GAPDH levels during the time course in four patients who received chemotherapy and allo-BMT. Patient 1 received eight courses of chemotherapy and allo-BMT in the 7-month period following the onset of the disease. Hematological relapse after BMT was observed in the 13th month, and the level of the AML1-MTG8 expression was found to be 10^{-3}. After the patient received re-induction chemotherapy and DLI (4.9×10^8 T cells/kg), he developed acute GVHD (grade III) and the administration of prednisolone (60 mg/day p.o.) was started. Prednisolone was

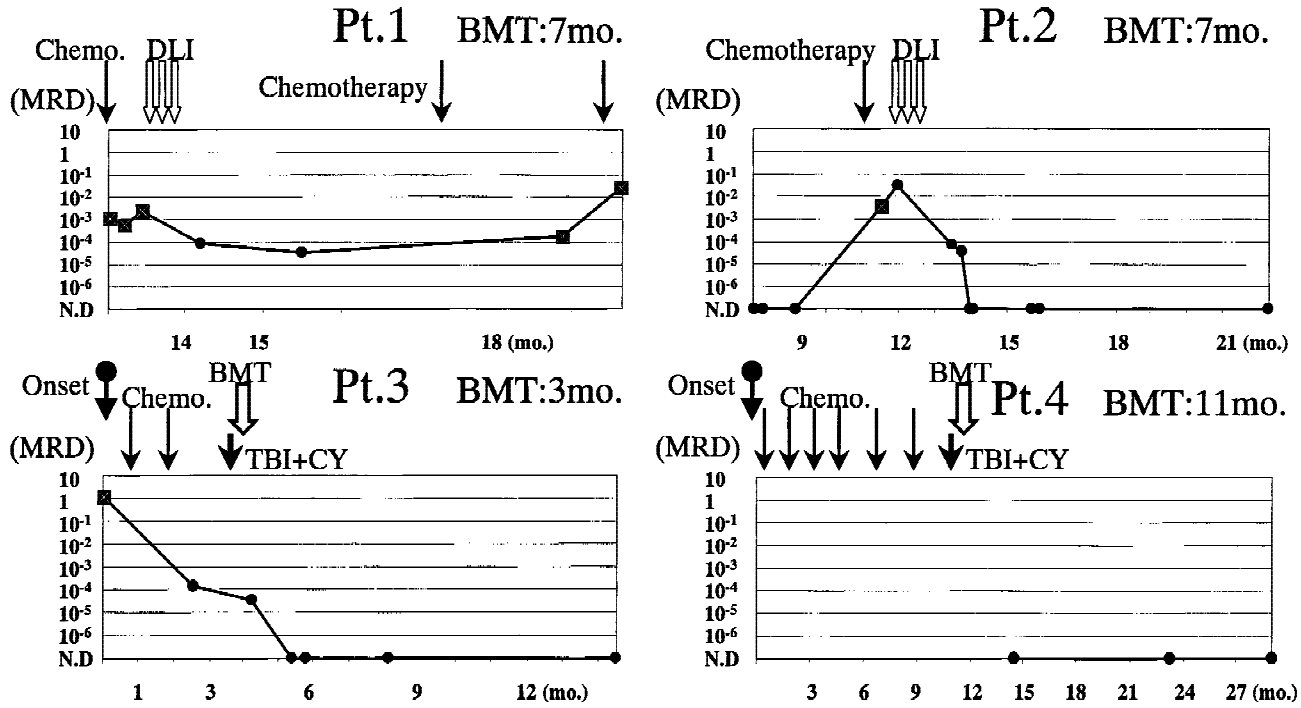

Fig. 2. Quantitation of MRD, expressed as the AML1-MTG8 transcript level in four patients that received chemotherapy and allo-BMT during the clinical course. The AML1-MTG8 transcript level normalized to GAPDH gene expression. The AML1-MTG8 transcript level is expressed as a fraction of the level of expression of undiluted SKNO-1 RNA. Expression levels of less than 10^{-6} were defined as N.D. (not detectable). DLI, donor lymphocyte infusion; BMT, bone marrow transplantation; TBI, total body irradiation; CY, cyclophosphamide. Stars indicate a relapsing state, and dots indicate a remission in hematological state. Hematological remission is defined as which blast cell percentage is less than five in a cellular marrow.

prescribed for 26 days and then tapered. Skin eruption was gradually decreased, and diarrhea was subsided. He became under the hematological remission state up to the 17th month with 10^{-4} and 10^{-5} levels of MRD. However, the disease relapsed again with AML1-MTG8 expression higher than the 10^{-2} level and he died soon after. Patient 2 received five courses of chemotherapy and allo-BMT in the 7-month period following the onset of the disease. The AML1-MTG8 mRNA level was under the detection limit until 2 months after BMT. Hematological relapse with an AML1-MTG8 expression higher than 10^{-2} was observed 4 months after BMT. She received re-induction chemotherapy and DLI (2.5×10^8 T cells/kg). Since severe acute GVHD of the skin and liver was complicated (grade III), she received steroid pulse therapy (mPSL 1 g/day × 3 days, 500 mg × 2 days, 250 mg × 2 days) followed by oral steroid therapy continued for 60 days. She then achieved hematological remission in which the AML1-MTG8 expression level rapidly decreased and became undetectable. In patient 3, the level of AML1-MTG8 mRNA was decreased from 1 to 10^{-4} with the achievement of hematological remission by the induction chemotherapy and one course of consolidation chemotherapy. Subsequently he received allo-BMT in the 3rd month and the AML1-MTG8 mRNA became undetectable within 3 months after BMT. Patient 4 received seven courses of chemotherapy and allo-BMT in the 11th month. His hematological remission state was maintained with all the measurements of the AML1-MTG8 mRNA expression below the detection level after 14th months.

AML1-MTG8 mRNA Levels in Patients Who Received Chemotherapy Alone

Figure 3 shows quantitative levels of AML1-MTG8 expression in the three patients who received chemotherapy alone. The clinical courses of these three patients were similar. The patients were treated with one induction chemotherapy, which lead to hematological remission in all cases, and then they received three courses of consolidation chemotherapy. As the clinical remission states continued, they continued to receive maintenance chemotherapy. The level of AML1-MTG8 expression was decreased in each case as a result of chemotherapy, but the expression levels remained detectable at 10^{-5} (patient 5), 10^{-6} (patient 6), and 10^{-6} (patient 7) in the final observation in spite of the hematological remission state.

DISCUSSION

The sensitivity of the real-time quantitative PCR assay seemed similar to that of the qualitative PCR as the 10^6-

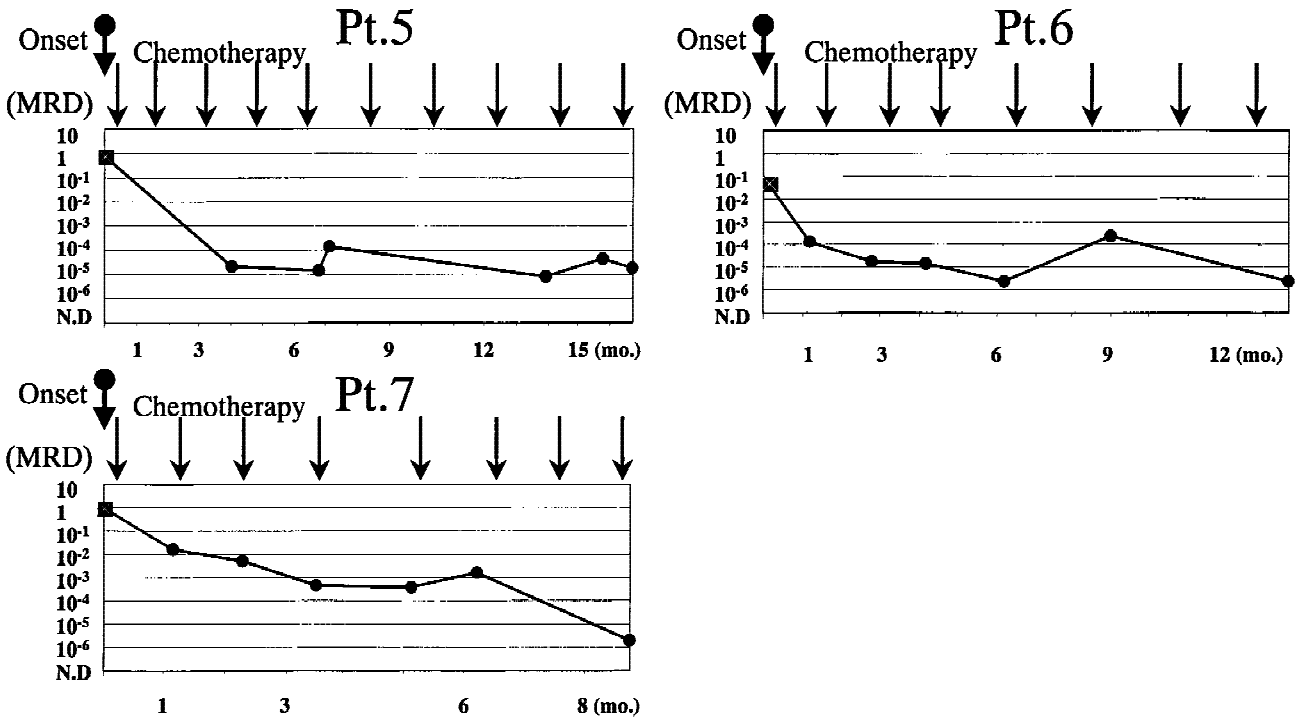

Fig. 3. Quantitation of MRD, expressed as the AML1-MTG8 transcript level in three patients that received chemotherapy alone. See Figure 2 for further details.

fold dilution of the positive control RNA could be detected (Fig. 1B) [17]. However, we found that samples from some of the patients that were undetectable in this assay were detectable by a nested PCR assay (data not shown). Thus, this real-time assay may be a little less sensitive than the nested-PCR assay. Levels of AML1-MTG8 mRNA in seven patients at different time points during the clinical course were generally correlated well with the clinical status of the patients (Figs. 2 and 3). Dynamic changes over 6 orders of magnitude in the levels of AML1-MTG8 mRNA could be observed in a single assay. The mRNA levels decreased after the therapies and increased during the relapsing phase. This result was analogous to the results obtained by a competitive PCR assay which has been reported to be useful in monitoring MRD of t(8;21)-positive AML [20,21].

The competitive PCR, however, is very time-consuming and cannot be used in a routine setting. The final results sometimes vary, which make them hard to assess and the use of the competitor DNA molecule is a potential source of contamination. In contrast, DNA amplification by the new method is detected in a closed tube, and no post-PCR sample handling is necessary, thus minimizing cross-sample contamination. The instrument provides real-time quantitative information and enables a high throughput of patient samples. The same system has been used successfully in the monitoring of Bcr-Abl mRNA levels in chronic myelogenous leukemia (CML) patients that received allo-BMT and DLT [26].

Using this system, we noted a decrease in the level of AML1-MTG8 mRNA down to the undetectable level in at least three patients treated by allo-BMT, but a similar decrease was not observed in the three patients treated by chemotherapy alone. A similar result was reported using qualitative PCR in which among 22 patients with t(8;21) AML in long-term remission states: AML1-MTG8 mRNA was not detected in four patients following allo-BMT, while the mRNA was detected in all 18 patients who received conventional chemotherapy only [16]. On the other hand, another report described the detection of AML1-MTG8 mRNA in samples from 10 patients with AML with t(8:21) in long-term remission after allo-BMT [17]. The discrepancy between the two reports may be due to the differences in the sensitivity of the qualitative PCR systems, although it is also possible that it was due to the differences in the treatment given to the patients. Our quantitative data, though preliminary, suggested that there may be a quantitative difference in the levels of MRD between these two therapeutic strategies (chemotherapy alone and chemotherapy combined with allo-BMT). Analysis of a larger number of patients by the quantitative assay will be necessary to clarify this point.

The greater reduction in the AML1-MTG8 mRNA, however, may only indicate a greater myelo-ablative activity of the regimen but it does not always mean a good prognosis for the patients. For example, patient 2 suffered a relapse of the disease just 2 months after this assay failed to detect AML1-MTG8 mRNA (Fig. 2). Thus, sequential analysis of the expression of AML1-MTG8 mRNA is definitely required to obtain practically

useful information concerning the prognosis. A decreasing or increasing tendency in mRNA levels can be a fairly reliable prognostic marker for the disease activity as reported in CML [26,27]. Since processing many samples with an appropriate internal control such as GAPDH can be done in a single assay within a short time, monitoring the kinetics of gene expression by this method is much easier and more accurate than any other existing methods at present. A long-term follow up by this method would be quite helpful in the detection of early relapse and in facilitating the decision of whether to offer additional treatment to the patients with t(8;21), regardless of the initial choice of therapeutic regimens.

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